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X. ANAPHYLATOXIN AND AMINO-NITROGEN

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SYNOPSIS

INTRODUCTION
TECHNIC
AMINO-NITROGEN CONTENT OF NORMAL SERUM
ANAPHYLATOXIN AND AMINO-NITROGEN
  AGAR ANAPHYLATOXIN
  DISTILLED WATER-RAT SERUM ANAPHYLATOXIN
  INULIN ANAPHYLATOXIN
  SENSITIZED SERUM + ANTIGEN
SUMMARY

The majority of workers who have concerned themselves with the study of anaphylatoxin believe that proteolysis is in some way responsible for the formation of this poison. Friedberger and his pupils insist that the matrix of the toxin lies in the antigen used. It is their notion that the complement of normal guinea-pig serum plays the part of a proteoclastic enzyme, digesting the proteins of the antigen with the resulting formation of toxic albumoses.

Nathan[1] demonstrated that normal guinea-pig serum could be toxified with starch, while Bordet produced typical anaphylatoxin by mixing fresh serum with 0.5% agar-hydrogel. Unlike the bacteria which Friedberger used as antigen, these substances contain minimal quantities of albuminoid nitrogen. Friedberger,[2] however, stubbornly maintained this might still be the source of the poison. Friedberger's objection was finally set aside by Kopaczewski and Mutermilch,[3] who produced anaphylatoxin by incubating guinea-pig serum with pectin, a nitrogen-free colloid. Bordet and Zunz[4] further clinched the matter by obtaining a like result with pararabine.

The view now arose that anaphylatoxin resulted from serum autolysis, and that the bacteria and foreign colloids introduced served merely to uncover a latent proteoclastic enzyme, which then proceeded to attack the proteins of the serum itself. This theory has been enunciated most clearly by Jobling and Petersen,[5] and by Bronfenbrenner.[6]

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[1] Ztschr. f. Immunitätsf., 1913, 18, p. 636.
[2] Ibid., 18, p. 323.
[3] Ibid., 1914, 22, p. 539.
[4] Ibid., 23, p. 42.
[5] Jour. Exper. Med., 1914, 19, p. 480.
[6] Ibid., 1915, 21, p. 480.

The former authors extracted normal guinea-pig serum over a period of from 12 hours to several days with chloroform or ether. They found that serum so treated became toxic in minute doses for homologous animals. It was their idea that these solvents removed lipoidal antiferments, with the result that a protease was set free. Their results are to be questioned seriously, since N. R. Smith[7] has shown that the method they employed does not free the serum entirely from the chloroform introduced, and that the residual amounts of chloroform are responsible for the toxic effects observed.

Bronfenbrenner incubated pregnant guinea-pig serum with boiled placenta and obtained a potent anaphylatoxin after 16 hours digestion. He tested the serum at the same time for dialyzable split products by the ninhydrin and biuret reactions. It appeared to him that the toxicity of the serum coincided with the appearance of these dialyzable bodies. Bronfenbrenner obtained first evidences of toxicity in 8 hours and found that maximal effect was to be observed in 16 hours. If this time were necessary for anaphylatoxin-production, it might be supposed that a ferment was concerned in the reaction. Such, however, is not the case. The production of anaphylatoxin may occur with extreme rapidity, and it is just this fact that makes it difficult to look upon this reaction as one of proteolysis.

Bordet and Zunz[8] recognized the value of the Van Slyke nitrous acid method in the detection of proteolysis and applied it to the study of agar and pararabine anaphylatoxins. These investigators incubated guinea-pig serum for from 2 to 3 hours with these substances and observed some increase in the aliphatic amino-nitrogen. This increase was very small in the case of pararabine. In one instance, where the toxicity was very marked, an increase of only 0.9 mg. per 100 c.c. was observed. Bordet and Zunz show proper caution in the interpretation of their results. They conclude that the proteolysis may as well be a concomitant as a causative phenomenon.

The method of Van Slyke[9] furnishes by far the most delicate and accurate instrument for determining protein cleavage, and we have utilized it exclusively in the experiments presented below. While it is plausible that there might be proteolysis in serum incubated over a period of 16 hours, it might be doubted that such a change could be detected in a few minutes. It has been our purpose to produce anaphylatoxin at a maximal speed, and to study any changes in quantity of serum amino-nitrogen that might accompany such production.

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[7] Jour. Lab. and Clin. Med., 1916, 1, p. 584.

[8] Ztschr. f. Immunitätsf., 1914, 23, p. 49.

[9] Jour. biol. Chem., 1912, 12, p. 275; 1913, 16, p. 121; 1915, 23, p. 407.

TECHNIC

The micro-apparatus of Van Slyke was used in all the determinations made. The serum-proteins were removed by precipitation with 10 volumes of 95% alcohol. To insure complete precipitation, this mixture was allowed to stand from 12 to 24 hours at the temperature of the room. The precipitate was then filtered off by suction and subsequently washed with a little alcohol. The washings were joined to the first filtrate. If the filtrate was not clear, as sometimes happened in the precipitation of dilute sera, gravity filtration was substituted for that by suction. The alcohol of the filtrate was then distilled off under reduced pressure at a boiling point of 38 C. and the water remaining was evaporated to dryness in a current of warm air, care being taken to avoid complete desiccation. The residue was taken up in 2 c.c. of distilled water and the determination made at once. Correction was made for nitrogen of reagents, for barometric pressure and for temperature. The reading on the gas-buret was then calculated to mg. of nitrogen per 100 c.c. of serum.

AMINO-NITROGEN CONTENT OF NORMAL SERUM

A series of preliminary determinations were made with normal rat serum, obtained by bleeding under ether with heart pipettes, immediate defibrination with a glass rod, and centrifugation of the clot-free blood at 8000 r.p.m. The same method was employed with all the material used in this work. The results of the series of preliminary tests with 7 different pools of serum are given in Table 147.

TABLE 147

AMINO-NITROGEN CONTENT OF NORMAL RAT SERUM

No.	Source	Amount of Serum Used (c.c.)	Amino-Nitrogen per 100 c.c. (mg.)
1	Rat	10	4.304
2	Rat	5	4.411
3	Rat	10	4.568
4	Rat	10	4.000
5	Rat	10	3.541
6	Rat	10	4.304
7	Rat	5	3.328

In the case of normal guinea-pig serum, we have found the variation to be slightly greater, that is, from 2 to 5 mg. per 100 c.c. The variation has no significance in subsequent experiments, since determinations on anaphylatoxic serum were invariably controlled by normal serum from the same pool.

ANAPHYLATOXIN AND AMINO-NITROGEN (AGAR ANAPHYLATOXIN)

Eight rats were bled in rapid succession, the blood defibrinated, pooled and centrifugated at 8000 r. p. m. It was iced for 1 hour before the experiment.

Five-tenths gm. of agar was dissolved in 100 c.c. of distilled water and autoclaved at 120 C. for 5 minutes, then gelled in cracked ice. The serum was then divided into 2 parts and treated as follows:

I. 14 c.c. serum, 38 C. + 3.5 c.c. agar-gel, 38 C., mixed for 1 min., incubated at 38 C. for 5 min., centrifuged at 3000 r. p. m. for 10 min.

Supernatant divided... { (a) 10 c.c. + 10 vol. alc., for Van Slyke
(b) Balance tested for toxicity

II. 12 c.c. serum, 38 C. + 3 c.c. 0.85% NaCl, incubated at 38 C. for 5 min.

Mixture divided...... { (a) 12.5 c.c. + 10 vol. alc., for Van Slyke.
(b) Balance tested for toxicity

The result is presented in Table 148.

TABLE 148

Amino-Nitrogen Content of Agar-Rat Serum: 5 Min. Incubation

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. Serum + agar.......	5	180	1.0	3′20″	1.683
II. Serum control.......	5	192	1.0	Nil	4.07

It will be seen that while the portion treated with agar became toxic after 5 minutes' incubation, it showed actually less amino-nitrogen than the control. Duplicates of this test invariably gave the same general result, that is, a lowering of the amino-nitrogen content of the anaphylatoxin as compared with the control.

An attempt was now made to duplicate this result with guinea-pig serum. In this case, the incubation with agar was prolonged to 15 minutes, since guinea-pig serum has not the lability of that of the rat.

Three guinea-pigs were bled in the usual way, the blood pooled and centrifugated. It was used at once after centrifugation. Thirty c.c. of the pooled serum were divided into 2 portions, as follows:

I. 20 c.c. guinea-pig serum + 4 c.c. agar-gel were mixed 1 min. at room temperature, placed at 38 C. for 15 min., transferred at once to centrifuge and swung for 10 min. at 3000 r. p. m.

Supernatant divided... { (a) 10 c.c. precipitated for Van Slyke.
(b) Balance tested for toxicity

II. 10 c.c. guinea-pig serum + 2 c.c. 0.85 % NaCl incubated at 38 C. for 15 min., mixture precipitated at once for Van Slyke.

TABLE 149

AMINO-NITROGEN OF AGAR-GUINEA-PIG SERUM; FIFTEEN MINUTE CONTACT

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. Serum + agar.......	15	190	3.0	3′5″ Typical	1.925
II. Serum control.......	15	—	—	—	2.975

The result in the case of the guinea-pig serum will be seen to be analogous to that of rat serum, although the drop in the amino-acid of the anaphylatoxin is not as great as in the latter. In another experiment of a similar nature, the toxic serum gave a value of 2.663 as against 4.26 mg. per 100 c.c. for the control.

The drop in the amino-acid content of the anaphylatoxin as compared with the normal serum control occurs within 5 minutes after the time the agar is mixed with the serum. It would be interesting to note whether the same result would follow mixture and immediate centrifugation. The following experiment shows that this is the case. It indicates that mere mixing of the guinea-pig serum with agar-gel, without incubation, gives rise to a noticeable amount of anaphylatoxin.

I. Two preliminary control injections of normal serum.

II. 20 c.c. guinea-pig serum, room temperature + 4 c.c. agar-gel, room temperature, mixed 1 min., centrifugated 5 min.

Supernatant divided... { (a) 10 c.c. + 10 vol. alc., for Van Slyke
(b) Balance tested for toxicity

III. 10 c.c. guinea-pig serum + 2 c.c. 0.85 % NaCl stood room temperature 5 min., + 10 vol. alc. for Van Slyke

TABLE 150

AMINO-NITROGEN CONTENT OF AGAR GUINEA-PIG SERUM; NO INCUBATION

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. Serum control......	0	193	4.0	Nil	
	0	192	4.0	Nil	
II. Serum + agar......	0	200	4.0	56″. Typical	2.39
	0	195	4.0	Severe shock	
III. Normal serum + NaCl.............	0	—	—	—	3.82

Neither incubation nor any appreciable length of time is necessary to produce the amino-acid decrease in agar-treated serum. Again, the reaction of toxin-production is almost instantaneous, since the serum-agar mixture began to centrifuge within 1 minute after the 1-minute agitation in the flask. Kopaczewski and Mutermilch[10] obtained a similar result. They, too, used guinea-pig serum with agar as the toxin inducer. The dose used in their experiments was 4.5 c.c. These authors observed severe shocks with such doses, even when the serum and agar were separately iced before mixture.

The results given were duplicated several times. When the agar and serum were separately incubated at 38 C. before mixture, the drop in amino-nitrogen was just as marked. It is worthy of note that the serum in this case was no more toxic, indeed even less so, than in the experiment tabulated above. If an enzyme were concerned in the toxin-production, one would expect the reverse to be true.

It was thought desirable to extend the preceding experiment with the addition of a sample incubated for 5 minutes at 38 C. The toxicity of such a mixture might well be greater than that of one not incubated. On the other hand, the foregoing tests would make it seem doubtful that there would be any parallel rise in amino-nitrogen. These expectations are shown to be justified by the following experiment.

A fresh pool of 55 c.c. of normal guinea-pig serum was used, together with the usual 0.5% agar-hydrogel.

I. 10 c.c. guinea-pig serum + 2 c.c. 0.85 % NaCl, (both room temperature), stood at room temperature 6 min., + 10 vol. alc. for Van Slyke

II. 20 c.c. guinea-pig serum + 4 c.c. agar-gel, (room temperature) mixed 1 min., centrifugated at 3000 r. p. m. for 6 min.

Supernatant divided... { (a) 10 c.c. + 10 vol. alc., for Van Slyke
(b) Balance tested for toxicity

III. 25 c.c. guinea-pig serum + 5 c.c. agar-gel, (room temperature) mixed 1 min., 38 C. 5 min., centrifugated for 6 min.

Supernatant divided... { (a) 10 c.c. + 10 vol. alc., for Van Slyke
(b) Balance tested for toxicity

The result of this experiment is given in Table 151.

This experiment demonstrates that a marked rise in toxicity may occur with no corresponding rise in amino-nitrogen. On the contrary, the amino-nitrogen of the sample incubated for 5 minutes at 38 C. is slightly lower than in the case of the nonincubated agar-serum mixture.

We do not know the cause of this decrease in amino-nitrogen in the agar treated serum, although it seems most probable that some of the

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[10] Compt. rend. Soc. de biol., 1914, 77, p. 392.

TABLE 151

Amino-Nitrogen Content of Agar Guinea-Pig Serum; 5 Minutes' Incubation

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. Normal serum + NaCl............	—	—	—	—	4.16
II. Serum + agar......	0	200	4.0	Slight	3.66
	0	204	4.0	Slight	
III. Serum + agar......	5	198	4.0	4′ Typical	3.27
	5	200	4.0	Severe shock	

amino-acid may be adsorbed by the agar-gel. It has been shown by Hamburger[11] that agar can adsorb enzymes, and we have good reason to believe that serum proteins may also be in part taken up by this substance.

We have attempted to recover amino-acids from the precipitate of agar thrown down by centrifugation of the agar-serum mixtures. Our procedure was to take up the agar sediment in distilled water by means of a bulb pipette, shake the mixture vigorously for 10 minutes, and digest it at 38 C. for one-half hour. The mixture was then re-centrifugated at 8000 r.p.m. to remove all traces of agar, and the supernatant was concentrated in a current of warm air. Ten volumes of 95% alcohol were added to the concentrate. The fairly heavy flocculent precipitate which resulted showed the presence in the fluid of a considerable amount of serum protein. The usual technic of filtration and concentration was then carried out, followed by the Van Slyke determination. No amino-nitrogen was evolved.

It is possible that if there is an adsorption of amino-acid by the agar, the combination is so firmly fixed that distilled water will not break it.

Since Bordet and Zunz[8] observed a distinct increase in amino-nitrogen, after incubating guinea-pig serum with agar for 3 hours at 38 C., we considered it likely that the preliminary drop might be followed by a rise if the incubation were continued. Experiments were made to detect such an increase.

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[11] Arch. nécr. d. sc. exact. et nat., 1908, 13, p. 428.

A pool of 40 c.c. of guinea-pig serum was divided into 2 portions and tested as follows:

I. 20 c.c. guinea-pig serum, 38 C., + 4 c.c. agar-gel, 38 C., incubated for 2 hr., 30 min., at 38 C., then centrifugated at 3000 r. p. m. for 10 min.

Supernatant divided... { (a) 10 c.c. + 10 vol. alc., for Van Slyke
(b) Balance tested for toxicity

II. 15 c.c. guinea-pig serum, 38 C., + 3 c.c. 0.85% NaCl, 38 C., 2 hr., 30 min.

$\frac{\text{12 c.c. of mixture}}{\text{10 c.c. actual}}$ + 10 vol. alc., for Van Slyke

The result of the experiment is given in Table 152.

TABLE 152

Amino-Nitrogen Content of Guinea-Pig Serum; Two Hours, Thirty Minutes' Incubation

Treatment of Serum	Time at 38 C.	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. Serum + agar.......	2 hr. 30′	195	2.0	Near-kill	3.497
II. Serum + NaCl......	2 hr. 30′	—	—	—	3.579

In this case the amount of amino-nitrogen is distinctly greater, as compared with the control, than in the experiments where short agar contact was employed. It would be too much to conclude that a distinct rise in amino-nitrogen had occurred, since no brief contact sample was available in this experiment. An experiment which should include the latter test was therefore made.

A pool of fresh guinea-pig serum was made and divided into 4 parts as follows:

I.—1. 20 c.c. guinea-pig serum, 38 C., + 4 c.c. agar-gel, 38 C., incubated at 38 C., 15 min., then centrifugated at 3000 r. p. m. for 10 min.

Supernatant divided... { (a) 10 c.c. + 10 vol. alc., for Van Slyke
(b) Balance tested for toxicity

2. 15 c.c. guinea-pig serum, 38 C., + 3 c.c. 0.85% NaCl, 38 C., mixture 38 C., 15 min.

$\frac{\text{12 c.c. of mixture}}{\text{10 c.c. serum}}$ + 10 vol. alc., for Van Slyke

II.—1. 20 c.c. guinea-pig serum, 38 C., + 4 c.c. agar-gel, 38 C., incubated at 38 C., 3 hr., 30 min., centrifuged at 3000 r. p. m., 10 min.

10 c.c. of supernatant + 10 vol. alc., for Van Slyke

2. 15 c.c. guinea-pig serum, 38 C., + 3 c.c., 0.85 % NaCl, 38 C., incubated at 38 C., 3 hr., 30 min.

$\frac{\text{12 c.c. of mixture}}{\text{10 c.c. serum}}$ + 10 vol. alc., for Van Slyke

The result of this experiment is given in Table 153.

TABLE 153

AMINO-NITROGEN CONTENT OF AGAR GUINEA-PIG SERUM; LONG AND SHORT INCUBATION

Treatment of Serum	Time at 38 C.	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. (a) Serum + agar	15′	202	3.0	3′45″	3.715
	15′	210	2.0	4′30″	
(b) Serum + NaCl	15′				3.23
II. (a) Serum + agar	3 hr. 30′				4.085
(b) Serum + NaCl	3 hr. 30′				4.013

This experiment shows an increase of 0.3 mg. per 100 c.c. in the agar-treated serum, while the control shows an increase of 0.8 mg. over the sample incubated for 15 minutes. It would appear, then, that increase in amino-nitrogen was hardly appreciable, even when the incubation was carried on for from 2 to 3 hours.

A more complete experiment of this kind was now performed, in which incubations were made over a period of 24 hours. The entire experiment was carried out with rigid aseptic precautions. A single pool of 160 c.c. of serum was used. The successive samples of the agar-treated serum and of the controls were removed, each from its flask, at the intervals indicated in outline of experiment.

I. 10 c.c. guinea-pig serum + 10 vol. 95 % alc., for Van Slyke

II. 50 c.c. guinea-pig serum + 10 c.c. 0.85 % NaCl, 38 C., incubation in sterile flask

12 c.c. portions
——————— removed at 15 min., 4 hr., 8 hr., 12 hr., 24 hr. each +
10 c.c. serum 10 vol. 95 % alc., for Van Slyke

III. 100 c.c. guinea-pig serum + 20 c.c. agar-gel, 38 C., incubation in sterile flask

24 c.c. portions
——————— removed at 15 min., 4 hr., 8 hr., 12 hr., 24 hr., each
20 c.c. serum centrifuged 3000 r. p. m., 6 min.

Supernatant divided... { (a) 10 c.c. + 10 vol. alc., for Van Slyke
(b) 10 c.c. for m. l. d. tests

The only inconsistency in this series of determinations is to be found in the 12 hour test of II. Here we found an unaccountable drop of 2 mg. per 100 c.c. This is probably due to some unobserved technical error. The conclusion to be drawn from this series of tests reveals the usual early slight decrease of the amino-nitrogen of the

TABLE 154

AMINO-NITROGEN CONTENT AND TOXICITY; AGAR GUINEA-PIG SERUM; TWENTY-FOUR-HOUR INCUBATION

Treatment of Serum	No.	Time at 38 C.	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. Serum control		0	—	—	—	4.28
II. Serum + NaCl	1	15′	—	—	—	3.90
	2	4 hr.				4.00
	3	8 hr.				4.55
	4	12 hr.				2.42*
	5	24 hr.				4.87
III. Serum + agar	1	15′	205	2.0	3′	3.47
	2	15′	210	1.0	Slight	
	3	15′	205	1.5	Severe	
	4	4 hr.	172	1.5	Slight	3.44
	5	4 hr.	200	2.0	Fair	
	6	4 hr.	202	2.5	Fair	
	7	4 hr.	195	3.0	3′40″	
	8	8 hr.	210	2.5	3′	3.25
	9	8 hr.	212	2.0	4′40″	
	10	8 hr.	202	1.5	Near-kill	
	11	8 hr.	180	1.5	Very severe	
	12	12 hr.	190	1.5	Fair	3.58
	13	12 hr.	195	2.0	3′30″	
	14	12 hr.	190	2.0	Severe	
	15	24 hr.	200	2.0	Light	3.85
	16	24 hr.	175	3.0	3′	

* Drop due to technical error.

anaphylatoxin as compared with the control. There is a slight rise in the values of both control and toxin in 24 hours, but the amino-nitrogen of the anaphylatoxin never attains the amount of that of the control. On the other hand, the rise in toxicity is very sudden, practically attaining its maximum in 15 minutes. It is possible that there is a slight increase in the 8-hour test (III, 10), but this apparent increase in lethal power may as well be due to the use of a hypersusceptible guinea-pig. Chart 17 indicates the relationship between toxicity and amino-acid content of anaphylatoxin.

ANAPHYLATOXIN AND AMINO-NITROGEN (DISTILLED WATER-RAT SERUM ANAPHYLATOXIN)

Agar-hydrogel was used as the inducing agent in all the preceding experiments. In Part IX, it has been shown that rat serum can be made anaphylatoxic by simple dilution with distilled water, accom-

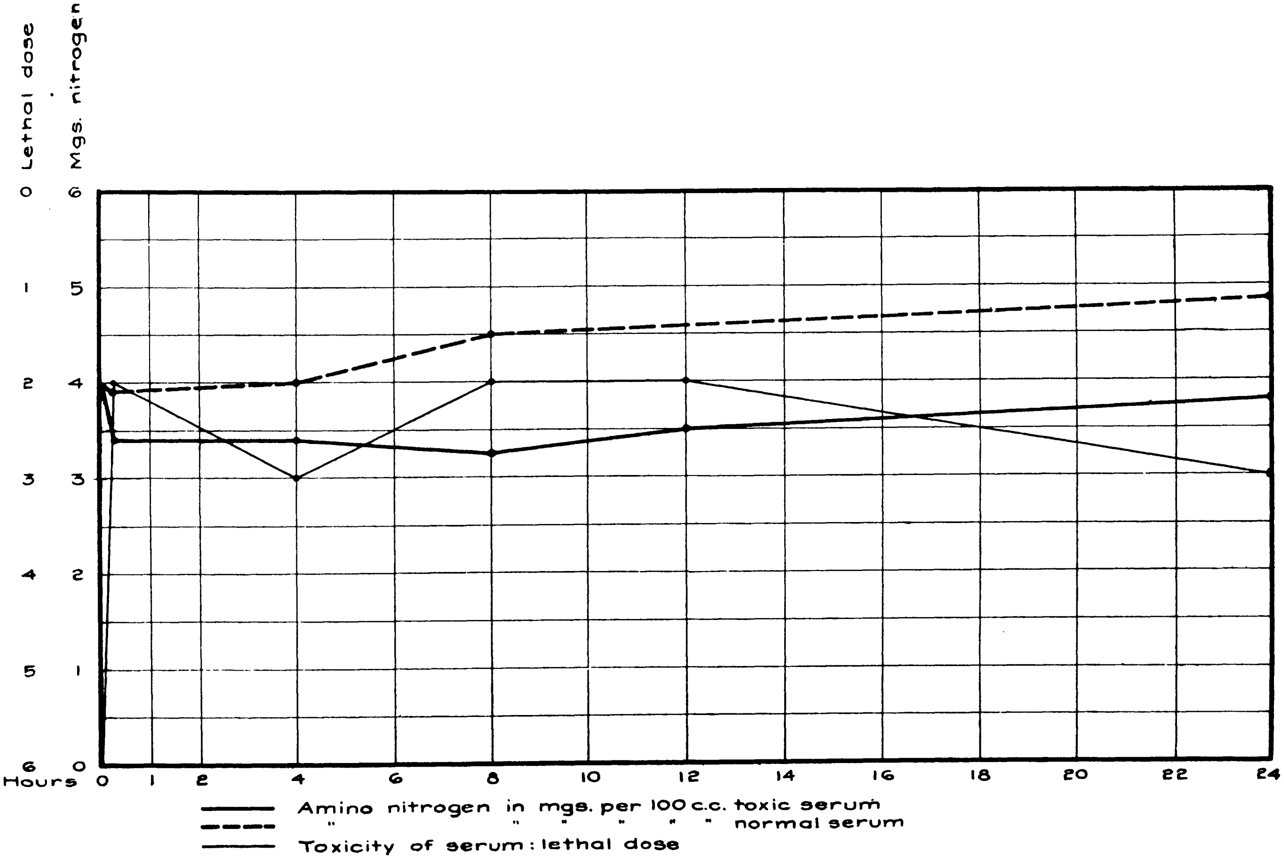

Chart 17.—Relation of Amino-nitrogen content to the toxicity of the Agar Guinea-pig serum (Table 154).

panied by incubation at 38 C. Sensitized rat serum is especially easy to toxify by this method, although similar results can be obtained by the use of normal serum. The only difference between the two lies in the fact that the former toxifies with greater rapidity. For example, if the serum of an egg-white sensitive rat is diluted in a proportion of 1:6 with distilled water, it becomes fatally toxic after 5 minutes at 38 C. Normal serum diluted in a similar manner requires 30 minutes of incubation to attain this toxicity.

Parallel determinations of toxicity and amino-nitrogen would be especially interesting in this type of anaphylatoxin-production, since no foreign element is introduced, the dilution being the only change. The injections of the diluted sera were made rather slowly (from 30 to 60 seconds), since too rapid injections of fluids of a low electrolyte content have a disturbing influence. Very severe effects might follow the sudden introduction of 6 c.c. of distilled water into the circulation of a 200-gm. guinea pig.

In the protocols presented, sensitive rat serum will be spoken of as 'rat>e.w. serum.' All the sensitive serum used in these experiments was obtained by intravenous injection of rats with 1% solutions of Kahlbaum's egg-white. Sensitization of the rats was carried out 38 days before exsanguination. The sera were pooled before use.

I. (a) 4 c.c. rat > e. w. serum + 24 c.c. distilled H_2O mixed 15 sec., + 10 vol. 95 % alc. for Van Slyke (control)
(b) 1 c.c. rat > e. w. serum + 6 c.c. distilled H_2O mixed 15 sec., injected at once (control)
(c) 1 c.c. rat > e. w. serum, 38 C., + 6 c.c. dist. H_2O, 38 C. mixed 15 sec., inc. 38 C., 5 min., injected at once
(d) 4 c.c. rat > e. w. serum, 38 C., + 24 c.c. dist. H_2O, 38 C. mixed 15 sec., 38 C., 5 min. + 10 vol. alc., Van Slyke

TABLE 155

Amino-Nitrogen Content of Diluted Sensitized Rat Serum

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. (a) Serum + H_2O	0	—	—	—	2.03
(b) Serum + H_2O	0	185	1.0	Nil	
(c) Serum + H_2O	5	187	1.0	3′10″	
(d) Serum + H_2O	5	—	—	—	3.45

The toxicity will be seen to have reached a 1 c.c. intensity after 5 minutes at 38 C. At the same time a rise of 1.4 mg. per 100 c.c. in amino-nitrogen took place. At first glance, one might infer that the toxicity was related to this rise. The following experiment shows that this is not true.

The conditions of the previous experiment were duplicated with the exception that pooled normal rat serum was substituted for the rat>e.-w. serum. Further, successive mixtures of the normal serum and distilled water were incubated over increasing lengths of time until the killing power of the mixtures attained a strength of 1 c.c. serum equivalent. When this incubation time had been ascertained, the Van Slyke mixture (C) was incubated for a corresponding length of time. Here, again, as in the preceding tests, the injections were made very slowly.

I.. (a) 4 c.c. normal rat serum + 24 c.c. distilled H_2O mixed 15 sec., + 10 vol. 95 % alc., Van Slyke
(b) 1 c.c. normal rat serum + 6 c.c. dist. H_2O mixed 15 sec., injected at once, control
(b_1) 1 c.c. normal rat serum, 38 C. + 6 c.c. dist. H_2O, 38 C., mixed 15 sec., 38 C., 10 min., injected
(b_2) like (b_1), 20 min. 38 C.
(b_3) like (b_1), 30 min. 38 C.
(c) 4 c.c. normal rat serum + 24 c.c. distilled H_2O, mixed 15 sec., 38 C., 30 min., + 10 vol. alc., Van Slyke

TABLE 156

AMINO-NITROGEN CONTENT OF DILUTED NORMAL RAT SERUM

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. (a) Serum + dist. H_2O	0	—	—	—	3.655
(b) Serum + dist. H_2O	0	200	1.0	Nil	
(b_1) Serum + dist. H_2O	10	200	1.0	Slight	
(b_2) Serum + dist. H_2O	20	195	1.0	Fair	
(b_3) Serum + dist. H_2O	30	198	1.0	3′15″	
(c) Serum + dist. H_2O	30	—	—	—	2.842

While an increase in amino-nitrogen occurred after incubation of the diluted rat>e.w. serum, a decrease will be observed to have taken place in the normal serum similarly diluted and incubated to a 1 c.c. killing power. These results are not fortuitous or due to technical variation. For in another series of tests, the amino-nitrogen of a rat>e.w. serum diluted 1:6 gave a figure of 5.51 before incubation, and 6.81 after 10 minutes at 38 C. A control of similarly diluted normal serum gave 6.09 before incubation, and 4.75 after 10 minutes at 38 C.

We think it desirable to extend these experiments and plan to do so as soon as a considerable supply of large rats becomes available. The present experiments are not extensive enough to warrant an explanation of this peculiar difference between normal and sensitive serum. They do show, however, that toxification does not depend on small increases or decreases in amino-nitrogen content.

ANAPHYLATOXIN AND AMINO-NITROGEN (INULIN ANAPHYLATOXIN)

Nathan[12] has demonstrated that the polysaccharid, inulin, produces anaphylatoxin in normal guinea-pig serum and that its ability to do so depends upon its physical state. An inulin-suspension produces the toxin readily, while a solution of the same substance fails to initiate such toxicity. The suspensions used in the following work were prepared by rubbing up, in the cold, 5 gm. of Kahlbaum's inulin with 100 c.c. 0.85% NaCl solution.

The experiments are, in general, of the same type as those made with agar-hydrogel. Toxicity tests of the serum were made parallel to the Van Slyke determinations. Before proceeding with the anaphylatoxin experiments, Van Slyke determinations were made of the inulin-suspension. This was found to contain an appreciable amount of amino-nitrogen. The procedure used in these determinations was identical with that in the serum work. A series of tests gave an average of from 3 to 4 mg. per 100 c.c. for 5% inulin-suspensions.

Although the amounts of inulin suspension used in anaphylatoxin-production were small, the amino-nitrogen content could not be neglected. Consequently, the values are expressed in terms of total nitrogen per 100 c.c. of mixture, rather than per 100 c.c. of serum.

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[12] Ztschr. f. Immunitätsf., 1914, 23, p. 204.

I. 40 c.c. guinea-pig serum + 4.0 c.c. 5 % inulin, mixed 1 min.

(a) $\frac{\text{5.5 mixture}}{\text{5 c.c. serum}}$ removed, centrifuged 3000 r. p. m., supernatant + 10 vol. alc., Van Slyke, balance at 38 C.

(b) After 30 min. at 38 C., 15 c.c. removed, centrifuged 3000 r. p. m.

Supernatant.......... $\begin{cases} \text{1. 5.4* + 10 vol. alc., Van Slyke} \\ \text{2. Balance tested for toxicity} \end{cases}$

(c) After 90 min. at 38 C., remainder centrifuged 3000 r. p. m.

Supernatant.......... $\begin{cases} \text{1. 5.4* + 10 vol. alc., Van Slyke} \\ \text{2. Balance tested for toxicity} \end{cases}$

* 5.4 c.c. is volume of supernatant when 5.5 c.c. are centrifuged, and is equivalent to 5.0 c.c. of serum.

The result of this experiment is given in Table 157.

TABLE 157

AMINO-NITROGEN CONTENT OF INULIN GUINEA-PIG SERUM

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. (a) Serum + inulin	0	—	—	—	4.59
(b) Serum + inulin	30 30	180 195	2.7 4.5	Nil 3′40″	
(b$_1$) Serum + inulin	30	—	—	—	6.49
(c) Serum + inulin	90 90 90	180 175 185	2.7 1.8 1.3	4′ 3′40″ Nil	
(c$_1$) Serum + inulin	90	—	—	—	5.95

After 30 minutes at 38 C., it will be noted that the toxicity is marked, but not at a maximum. Coincident with the toxin increase, the amino-nitrogen has risen 1.9 mg. per 100 c.c. of mixture. At the end of 90 minutes, at 38 C., the toxicity has increased to a great extent, 1.8 c.c. producing a fatal shock. The amino-nitrogen has at the same time dropped 0.5 mg. per 100 c.c. While there is a slight rise in amino-nitrogen, together with some toxicity, after an incubation of 30 minutes, the nitrogen increase fails entirely to keep pace with the intensification of the toxic state.

The rise noted in the case of the guinea-pig anaphylatoxin might lead one to conclude that at least the initiation of toxicity is accompanied by proteolysis. The following experiment with rat serum shows this to be untrue.

A pool of fresh rat serum was used in this experiment and a new suspension of inulin (5%) was made.

I. (a) 1 c.c. rat serum + 0.1 c.c. 5 % inulin mixed 30 sec., centrifugated at once, 3000 r. p. m., tested for toxicity
   (b) 4 c.c. rat serum + 0.4 c.c. 5 % inulin mixed 30 sec., centrifugated at once, 3000 r. p. m., supernatant + 10 vol. 95 % alc., for Van Slyke
II. (a) 2 c.c. rat serum, 38 C., + 0.2 c.c. inulin mixed 30 sec., placed at 38 C., 2 min., 30 sec., centrifugated 3000 r. p. m., 7 min., supernatant tested for toxicity
   (b) 4 c.c. rat serum, 38 C., + 0.4 c.c. inulin mixed 30 sec., placed at 38 C., 2 min., 30 sec., centrifugated 3000 r. p. m., 7 min., supernantant + 10 vol. 95 % alc., for Van Slyke
III. (a) 2 c.c. rat serum, 38 C., + 0.2 c.c. inulin mixed 30 sec., placed at 38 C. 7 min., centrifugated 3000 r. p. m., 7 min., supernatant tested for toxicity
   (b) 4 c.c. rat serum, 38 C. + 0.4 c.c. inulin mixed 30 sec., placed at 38 C., 7 min., centrifugated 3000 r. p. m., 7 min., supernatant + 10 vol. 95 % alc., for Van Slyke

The entire experiment covered less than an hour of time, so that any variation in amino-nitrogen, on account of time in I (b), II (b), or III (b), would be improbable.

The results are given in Table 158 and Chart 18.

TABLE 158

Amino-Nitrogen Content of Inulin Rat Serum

Treatment of Serum	No.	Time at 38 C.	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. Serum + inulin						
(a)	1	0	205	1.0	Nil	
(b)		0	—	—	—	3.02
II. Serum + inulin						
(a)	2	2′30″	205	1.0	4′	
	3	2′30″	190	0.5	Near-kill	
(b)		2′30″	—	—	—	2 67
III. Serum + inulin						
(a)	4	7′	206	0.5	3′25″	
	5	7′	185	0.25	3′30″	
	6	7′	193	0.20	Slight	
(b)		7′	—	—	—	2.73

It is necessary to place the mixture at 38 C. for but 2½ minutes to obtain a killing power of between 0.5 and 1.0 c.c. Despite this, the amino-nitrogen has fallen off distinctly. Furthermore, in the brief incubation time of 7 minutes, the serum has reached an extreme toxicity, yet the amino-nitrogen, III (b), is hardly larger in amount than that in II (b), and is certainly less than that in the control, I (b). See Chart 18.

To sum up, both guinea-pig and rat serum can be made anaphylatoxic by incubation with inulin suspension. Guinea-pig serum shows a slight early rise in amino-nitrogen, along with a moderate early toxicity. Subsequent intensification is not accompanied by a corresponding increase of amino-acid. The behavior of rat serum is totally different, and presents still stronger evidence of a dissociation of the phenomena of toxification and proteolysis. For while a maximum toxicity appears, the amino-nitrogen becomes less, as in the case of agar.

ANAPHYLATOXIN AND AMINO-NITROGEN (SENSITIZED SERUM + ANTIGEN)

Sensitive guinea-pig or rat serum can be made anaphylatoxic by incubation with the antigen used in sensitizing. This is also true of rabbit serum. We have produced anaphylatoxin in this manner with guinea-pig>egg-white and rabbit>egg-white sera and have made parallel tests of the amino-nitrogen. If a specific proteoclastic ferment were formed in response to the injection of a foreign protein, and if the toxicity subsequent to the bringing together of the serum of the injected animal, and the specific antigen were due to proteolysis of the latter, a large increase in amino-nitrogen should accompany the toxicity. The following experiments show that this is not the case. In fact, there is no evidence whatever of proteolysis, either of the serum or the antigen. At the same time, the serum in question becomes toxic to marked degree.

The rabbit used as source of the sensitive serum was made hypersensitive by 6 heavy intravenous injections of 4% egg-white, the doses ranging from 2 to 8 c.c. Kahlbaum's egg-white was used as an antigen in this experiment (1% in 0.85% NaCl solution).

I. Test of toxicity of rabbit > egg-white serum
II. (a) 2 c.c. rabbit > e.-w. serum + 0.2 egg-white, 38 C., 5 min.
(b) 2 c.c. rabbit > e.-w. serum + 0.2 c.c. egg-white, 38 C., 10 min.
(c) 2 c.c. rabbit > e.-w. serum + 0.2 c.c. egg-white, 38 C., 15 min.
(d) 2 c.c. rabbit > e.-w. serum + 0.2 c.c. egg-white, 38 C., 26 min.
(d_1) 2 c.c. rabbit > e.-w. serum + 0.2 c.c. egg-white, 38 C., 26 min.
III. (a) 4 c.c. rabbit > e.-w. serum + 0.4 c.c. egg-white, mixed 30 sec., mixture + 10 vol. 95 % alc., for Van Slyke
(b) Like (a), but incubated 38 C., 26 min. after mixture, then + 10 vol. alc.
Injection dose, 2.2 c.c. of mixture, equivalent to 2 c.c. of serum.

TABLE 159

Amino-Nitrogen Content of Rabbit > Egg-White Serum

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. Rabbit > e.-w. serum (control of toxicity)	0	205	1.0	Nil	
	0	190	2.0	Slight	
	0	206	2.5	Slight	
	0	206	3.0	Moderate	
II. Rabbit > e.-w. serum + egg-white	(a) 5	202	2.0	Nil	
	(b) 10	190	2.0	Fair	
	(c) 15	195	2.0	Fair	
	(d) 26	195	2.0	8′30″	
	(d_1) 26	196	2.0	Near-kill	
III. Rabbit > e.w. serum + egg-white	(a) 0	—	—	—	4.18
	(b) 26	—	—	—	2.003

The injections of sensitive serum not mixed with egg-white (I) serve as controls for the injections II (a), (b), (c), (d), and (d_1). The serum used, as would be expected from the many injections of egg-white made, had a very powerful precipitating action. For example, mixture II (a), after 5 minutes incubation, showed a heavy precipitate. It is possible that, here, as in the case of the agar-anaphylatoxin, the decrease in amino-nitrogen after incubation may be due to adsorption phenomena. The Van Slyke determinations, III (a) and (b) show clearly the absence of proteolysis in this case. At the same time, the toxicity of the serum egg-white mixtures attained a marked intensity after 26 minutes at 38 C. It may be remarked here that rabbit serum anaphylatoxin rarely reaches a higher concentration in regard to toxicity.

Similar experiments made with guinea-pig > egg-white serum yielded a like result. In this case, however, the guinea-pigs received but 1 injection of 1% egg-white. It required 60 minutes at 38 C. to toxify a mixture of 4 c.c. serum plus 1 c.c. of egg-white. A similar mixture, injected at once, without incubation, produced no effect. Its amino-nitrogen value was 1.35 mg. per 100 c.c. of mixture. Successive tests of 4 plus 1 mixtures were made after incubation at 5, 15, 30, and 60 minutes, respectively. These mixtures produced progressively heavier shocks, the last one (60 minutes) killing the recipient in 4 minutes and 10 seconds. The amino-nitrogen value of a 4 plus 1 mixture, incubated at 38 C. for 60 minutes was 1.25 per 100 c.c.

To sum up, a decrease rather than an increase in amino-nitrogen is noticed when sensitive sera are incubated with their specific antigens. It should be borne in mind that none of these mixtures are incubated over long periods. They were kept at 38 C. only long enough to acquire marked toxicity, since it was our object only to find whether such toxification would be accompanied by proteolysis.

Careful search has failed to disclose any evidence that would connect digestion of serum proteins with the formation of anaphylatoxin. In 2 or 3 instances, slight increases in amino-nitrogen have been detected at times when a serum was also anaphylatoxic. But in each of these cases, the use of another inducing agent with the same serum has given rise to toxicity without increase in amino-nitrogen (Tables 157 and 158). In cases where a moderate toxicity was accompanied by a slight amino-acid increase, intensification of that toxicity might occur with no further increase, indeed, at times, even a drop, in amino-nitrogen (Table 157).

Dale[13] has pointed out, in his work on the anaphylactic reaction of plain muscle, that the time relation of the reaction places a serious obstacle in the way of the ferment conception of anaphylaxis. The reaction is as rapid as in the case of application of a direct stimulant. We believe the toxification of normal serum to be a reaction identical with that occurring in the body cells and fluids during anaphylactic shock, and in the reaction studied, the same explosive effect is observed. In this connection, we wish to present an illustration of the time relation in anaphylatoxin-production.

Table 160 indicates the rise in toxicity of rat serum incubated with 5% inulin-suspension in a proportion 10: 1. The table is a composite of a series of experiments, all of which were made with the same pool of rat serum and the same suspension of inulin. The deaths were all acute.

TABLE 160

SPEED OF INCREASE IN TOXICITY OF INULIN RAT SERUM

No.	M.l.d. 200 gm. g.p.	Time at 38 C.
1	0.50	2′30″
2	0.25	7′
3	0.20	15′
4	0.15*	30′
5	0.20	60′
6	0.20	90′

* To our knowledge the most potent anaphylatoxin ever obtained.

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[13] Jour. Pharmacol. and Exper. Therap., 1913, 4, p. 167.

Chart 18 shows clearly the curve of toxicity. The degree of toxicity is plotted on the ordinates. The abscissae represent the time in minutes. The toxicity of the rat serum before mixture with the inulin was in all probability not less than 6 c.c. for a 200-gm. guinea-pig.

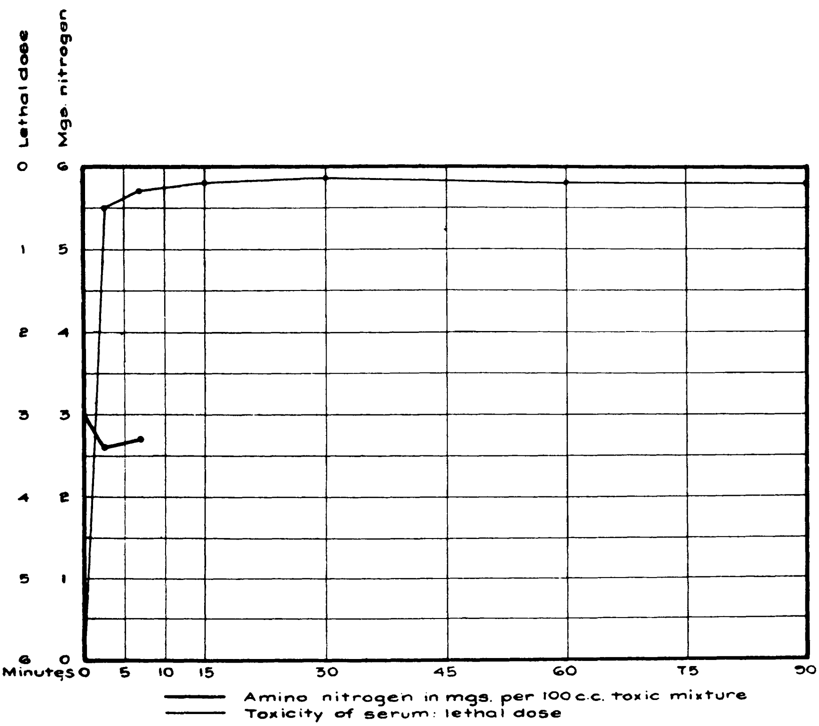

Chart 18.—Relation of Amino-nitrogen Content to the Toxicity of Inulin Rat Serum (Tables 158 and 160).

As Bayliss[14] points out, the digestion of raw serum by trypsin is very slow. It is a matter of hours before the reaction begins to become noticeable. Those who assume that a trypsin-like ferment is operating here will find it difficult to reconcile this curve with that characteristic of tryptic action in raw serum.

Table 161 gives a summary of the experiments with anaphylatoxin. In the majority of these, agar was used as the inducing agent; in others, inulin, distilled water, or antigen were employed.

[14] The Nature of Enzyme Action, 1914, p. 128.

TABLE 161

SUMMARY OF AMINO-NITROGEN VALUES IN NORMAL AND TOXIC SERA

No.	Source	Time at 38 C.	Lethal Dose per 200 gm. (c.c.)	Amino-Nitrogen per 100 c.c. (mg.)	
				Toxin	Control
1	Guinea-pig	0	4.0	2.39	3.82
2	Guinea-pig	0	—	3.66	4.16
3	Guinea-pig	1′	—	3.09	5.10
4	Guinea-pig	5′	—	3,27	4.16
5	Guinea-pig	15′	—	2.66	4.26
6	Guinea-pig	15′	3.0	1.92	2.97
7	Guinea-pig	15′	2.0	3.71	3.23
8	Guinea-pig	15′	2.0	3.47	3.90
9	Guinea-pig*	30′	4.5	6.49	4.59
10	Guinea-pig*	1 hr. 30′	1.8	5.95	4.59
11	Guinea-pig	2 hr. 30′	3.0	3.49	3.59
12	Guinea-pig	3 hr. 30′	3.0	4.08	4.01
13	Guinea-pig	4 hr.	3.0	3.44	4.00
14	Guinea-pig	8 hr.	2.0	3.25	4.55
15	Guinea-pig	12 hr.	2.0	3.58	2.42†
16	Guinea-pig	24 hr.	3.0	3.85	4.87
17	Rat*	2′30″	0.5	2.67	3.02
18	Rat	5′	1.0	1.68	4.07
19	Rat	5′	0.5	3.00	4.12
20	Rat*	7′	0.25	2.73	3.02
21	Rat‡	5′	1.0	3.45	2.03
22	Rat‡	10′	—	6.81	5.51
23	Rat‡	10′	—	4.75	6.09
24	Rat‡	30′	1.0	2.84	3.65
25	Rabbit§	26′	2.0	2.00	4.18
26	Guinea-pig§	60′	4.0	1.25	1.35

* Inulin. † Technical error. ‡ Distilled water. § Antigen.

SUMMARY

By the use of Van Slyke's method for the determination of aliphatic amino-nitrogen, we have attempted to discover a relationship between serum autolysis and anaphylatoxin-production. No relationship between these phenomena has been found.

Rat and guinea-pig sera, toxified rapidly by the addition of 5% agar-hydrogel, show a marked decrease in amino-nitrogen as compared with controls from the same pool of serum.

This decrease takes place at once upon addition of agar to serum. After this preliminary drop in amino-nitrogen, a very gradual rise to a value not exceeding that of the control may take place. Maximal toxicity appears before this increase begins to be noticeable.

The cause of this drop in amino-nitrogen in serum treated with agar is not known, but is probably to be referred to adsorption of amino-acid by the agar. Attempts to recover this supposedly adsorbed amino-acid have failed.

A similar drop in amino-nitrogen along with a marked increase in toxicity is to be observed in the case of rat serum incubated with inulin. Guinea-pig serum treated in a like manner shows a slight increase in amino-acids, which, however, does not keep pace with the increase in toxicity.

Rat serum behaves peculiarly when diluted with distilled water. Sensitive rat serum shows an increase, normal rat serum, a decrease in amino-nitrogen when incubated with 6 volumes of distilled water. Despite this difference, both become toxic.

Rabbit $>$ egg-white serum, mixed with egg-white, becomes toxic after a short time at 38 C., the amino-nitrogen at the same time decreasing by 50% as compared with the control made before incubation.

Inulin, mixed in proportion of 1:10 with normal rat serum produces the most potent anaphylatoxin yet recorded. The curve of toxicity shows no similarity to the usual picture of tryptic digestion of raw serum-protein.

ERRATA

Page 515. Last line, change 506 to 507.
" 542. 16th line from bottom, change 'kept' to swung.
" 569. 11th line from above, change Table 15 to Table 19.
" 576. In Table 36, change 0.024 to 0.048.
" 595. In Table 48, change dosage of No. 10 from 0.15 to 0.25.
" 606. In 2d line of foot-note to the table, change liks to like.
" 611. In 3d line from above change 1 hour to ½ hour.
" " In 14th line from bottom change "the inactivated" to 'tho inactivated.'
" 612. In 12th line from bottom, change Exper. 8 to Exper. 3.
" 614. In 2d line of foot-note to the table, change 8 and 9 to 3 and 4.
" 622. Delete the 5th line from above and insert: employed in the study of anaphylactic shock with excellent results.
" 629. In 5th line of synopsis, change sulution to solution.
" 630. In 2d line from above, change naphylatoxin to anaphylatoxin.
" 637. In 14th line from above change 'treated serum' to 'serum treated.'
" 647. In Table 66 under Transfer Time, change Min. to Sec.
" 651. " " 69 " " " " " " "